IFN-γ, IL-21, and IL-10 Co-Expression in Evolving Autoimmune Vitiligo Lesions of Smyth Line Chickens

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The Smyth line (SL) of chicken is an excellent animal model for human autoimmune vitiligo. In SL vitiligo (SLV), postnatal loss of melanocytes in feathers appears to be due to cell-mediated immunity. In this study, leukocyte infiltration and associated expression (RNA) of immune function-related cytokines in growing feathers were investigated throughout SLV development and progression. Both leukocyte infiltration and cytokine expression levels started to increase near visible SLV onset (early SLV), reached peak levels during active SLV, and decreased to near pre-vitiligo levels after complete loss of melanocytes. Specifically, significant increases were noticed in relative proportions of T cells, B cells, and major histocompatibility complex (MHC) II-expressing cells during active SLV. Levels of T-cell infiltration were higher than those of B cells, with more CD8+ than CD4+ cells throughout SLV. Elevated leukocyte infiltration in early and active SLV was accompanied by increased levels of cytokine expression, especially in IFN-γ, IL-10, and IL-21. Low expression of IL-4 and IL-17 did not suggest important roles of Th2 and Th17 cells in SLV pathogenesis. Taken together, SLV appears to be a Th1-polarized autoimmune disease, whereby IFN-γ expression is strongly associated with parallel increases in IL-10 and IL-21, particularly during early and active stages of SLV.

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INTRODUCTION

The mutant Smyth line (SL) of chicken is an excellent animal model for the study of autoimmune vitiligo (Wick *et al.*, 2006) due to many phenotypic and etiopathological similarities between SL and human vitiligo, and the multifactorial nature, high incidence, and spontaneous onset of SL vitiligo (SLV; Smyth, 1989; Erf, 2010). In chickens, melanocytes, the target cells in vitiligo, are located in growing feathers (Figure 1; Smyth, 1989). Growing feathers can be easily removed and can regenerate, and hence the developing autoimmune lesion can be monitored throughout SLV in the same individual. Moreover, the living portion of the growing feather (feather tip; Figure 1a) provides sufficient tissue sample for various post-collection analyses.

Similar to human autoimmune vitiligo, both humoral and cellular immunity have been implicated in SLV, with a more prominent role attributed to cellular immunity in melanocyte loss based on phenotypical analysis of infiltrating leukocytes (Erf *et al.*, 1995; Shresta *et al.*, 1997; Wang and Erf, 2004) and observation of IFN-γ expression in feather samples collected from SL chickens with active SLV (Wang, 2001; Shi *et al.*, 2009). Moreover, the presence of melanocytes-specific cell-mediated immunity was demonstrated *in vivo* based on the delayed wattle-swelling response to injection of syngeneic melanocyte lysates in chickens with SLV (Wang and Erf, 2003).

Although phenotypic analyses of infiltrating cells in cross-sections of active lesions and in feather pulp cell suspensions support a more prominent role of cell-mediated immunity in melanocyte loss in SLV (Erf *et al.*, 1995; Shresta *et al.*, 1997; Wang and Erf, 2004), immune functional activities associated with SLV development have not been examined. The objective of this study was to monitor cytokine gene expression and determine leukocyte infiltration profiles in feather tips (Figure 1a) collected over the course of SLV development from SLV chickens. Specifically, using two-step quantitative reverse transcriptase PCR, gene expression profiles were established for inducible nitric oxide synthase (iNOS) and cytokines of innate immunity (IL-1β, IL-6, IL-8, IL-10, IL-12α, IL-12β, and IL-15), signature cytokines of T helper (Th)1, Th2, and Th17 cells (IFN-γ, IL-4 and IL-17F, respectively), and IL-21, a pleiotropic cytokine implicated in organ-specific autoimmune diseases. To relate gene expression with leukocyte infiltration, the presence of macrophages and T- and B-lymphocyte subsets were also examined in longitudinal sections prepared from feather tips. Knowledge gained from this study will shed more light on the pathogenic mechanisms involved in the autoimmune loss of melanocytes in SLV.

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Abbreviations: iNOS, inducible nitric oxide synthase; MHC II, major histocompatibility complex II; SL, Smyth line; SLV, Smyth line vitiligo; Th, T helper

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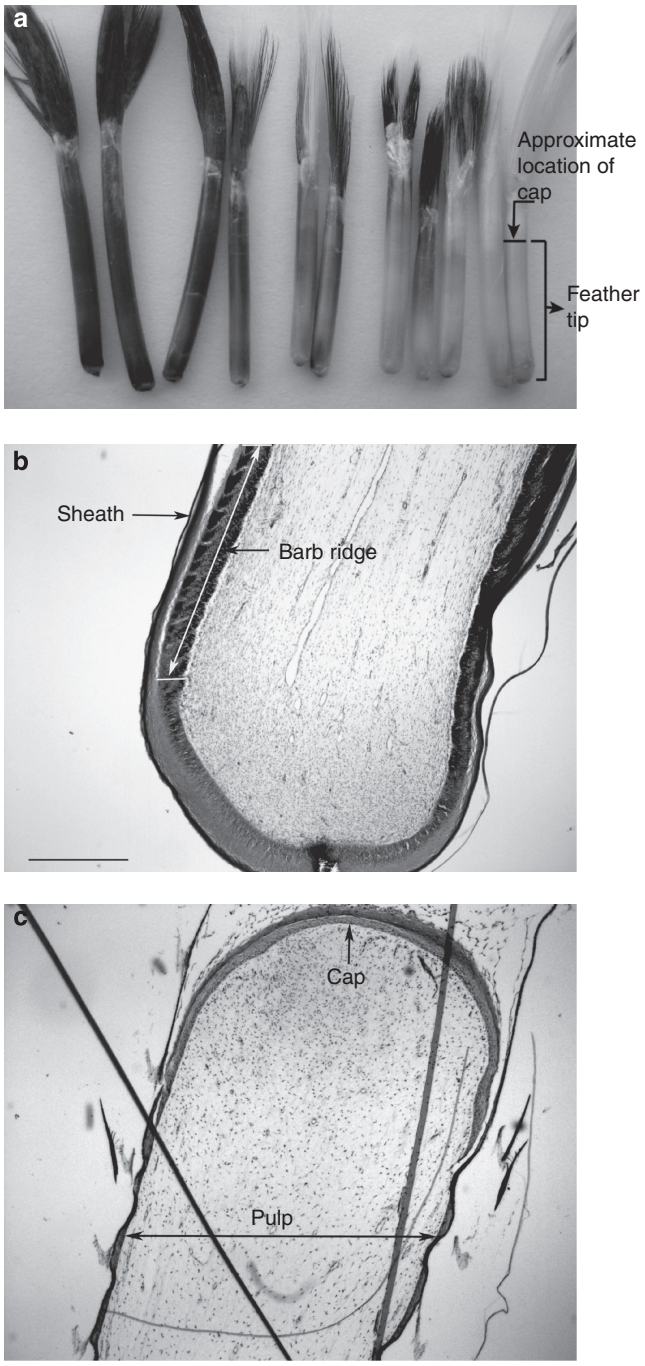

Figure 1. Morphology of 2- to 3-week-old growing feathers from SL chickens. (**a**) From left to right: normally pigmented, partially depigmented, and completely depigmented growing feathers from SL chickens that developed SLV. Growing feathers can be collected from SLV chickens over the whole course of SLV. The living part of growing feathers (newest growth to the epidermal cap) is referred to as ''feather tip''. (**b**) Microstructure of the newest growth of a feather tip with normal pigmentation; layers shown from the outside to inside are sheath, barb ridge, and pulp. (**c**) A cap formed by the epidermal layer enclosing the pulp. Longitudinal sections were stained with hematoxylin and eosin stain and examined at ×40 (**b**, **c**) original magnification under a bright-field microscope. Bar = 1 mm.

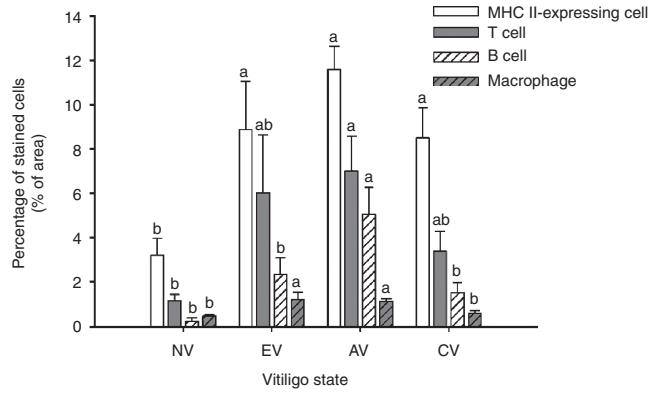

Figure 2. Infiltration profiles of leukocytes in feather tips collected at different states of SLV. Major histocompatibility complex (MHC) II-expressing cells, T cells, B cells, and macrophages were identified by indirect immunoperoxidase staining using Ia, TCR γδ and TCR αβ, Bu-1, and KUL01 mouse anti-chicken mAbs, respectively. Stained sections were examined at ×40 original magnification, and the amount of stained cells was expressed as the percentage of the whole feather section area analyzed (% of area). NV: feather tips from three SL chickens that never developed SLV; EV, AV, and CV: feather tips were collected from seven SLV chickens just before, during, and after SLV development, respectively. Each bar represents the mean ± SE. (a, b) For each cell type, means without common letters are different ($P \leqslant 0.05$).

RESULTS

Leukocyte infiltration in growing feathers collected at different states of SLV

For each cell type, the relative amount of staining was expressed as the percentage of the longitudinal sections analyzed (% of area). Leukocyte infiltration data from each individual were grouped with respect to vitiligo state: NV (feather tips from SL chickens that never developed vitiligo), EV, AV, and CV (feather tips from SLV chickens just before visible SLV onset, during active, and after complete depigmentation, respectively).

The infiltrating leukocytes consisted primarily of T and B lymphocytes, which reached peak levels during AV ($P \leqslant 0.05$). Although proportionately fewer macrophages than T and B lymphocytes were present in SLV feathers, macrophage levels were already elevated in EV and remained elevated in AV ($P \leqslant 0.05$; Figure 2). The relative proportions of major histocompatibility complex (MHC) II-expressing cells were higher than those of other leukocytes, independent of SLV state. Except for MHC II-expressing cells, the proportions of T cells, B cells, and macrophages returned to near NV levels when melanocyte loss was complete (CV, Figure 2).

Both types of TCR-defined T cells (αβ and γδ TCR) followed the same infiltration trend (Table 1). However, whereas the relative proportions of αβ T cells were greatly elevated ($P \leqslant 0.05$) in AV compared with NV samples, those of γδ T cells did not differ between NV, EV, AV, and CV samples. CD4+ and CD8+ cells reached peak levels in AV samples compared with NV, EV, and CV samples ($P \leqslant 0.05$, Table 1). At all SLV states, there were more CD8-expressing cells than CD4-expressing cells, with CD4:CD8 ratios being

Table 1. Relative proportions of leukocytes (% of area)[1] in feather tips[2] from SL chickens with and without SLV

	Vitiligo state[3]			
	NV	EV	AV	CV
T cells (αβ TCR+)	$0.70 \pm 0.17^{b,4}$	$3.95 \pm 1.57^{a,b}$	4.92 ± 1.10^{a}	$2.15 \pm 0.67^{a,b}$
T cells (γδ TCR+)	0.47 ± 0.12	2.08 ± 1.13	2.10 ± 0.57	1.25 ± 0.35
CD4+ lymphocytes	0.61 ± 0.30^{b}	2.35 ± 0.67^{b}	4.81 ± 0.48^{a}	1.49 ± 0.51^{b}
CD8+ lymphocytes	1.17 ± 0.52^{b}	3.51 ± 0.88^{b}	5.71 ± 0.64^{a}	2.37 ± 0.60^{b}
CD4+:CD8+	0.48 ± 0.06^{b}	$0.68 \pm 0.08^{a,b}$	0.87 ± 0.10^{a}	0.68 ± 0.13^{ab}
B cells (Bu-1+)	0.25 ± 0.15^{b}	2.37 ± 0.73^{b}	5.07 ± 1.21^{a}	1.53 ± 0.46^{b}
IgM+ cells	0.31 ± 0.05^{b}	1.89 ± 0.62^{ab}	4.30 ± 1.15^{a}	1.71 ± 0.48^{b}
T:B[5]	5.16 ± 2.66^{a}	$2.69 \pm 1.10^{a,b}$	1.52 ± 0.21^{b}	$2.94 \pm 0.69^{a,b}$

[1]Leukocyte types were determined by indirect immunoperoxidase staining. Primary unlabeled antibodies were mouse-anti-chicken mAbs to αβ TCR (cocktail of αβ1 and αβ2 TCR), γδ TCR, CD4, CD8, Bu-1 (B-cell marker) and IgM. Binding of primary antibodies was detected by biotinylated horse-anti-mouse IgG (H+L) with avidin-biotin peroxidase complex reagents and using 3,3-diaminobenzidine as the substrate. Image analysis was carried out at ×40 original magnification under a bright-field microscope. The amount of stained cells in feather tip sections was expressed as the percentage of the whole area analyzed (% of area).
[2]For each sample collection, three feather tips were laid parallel in the same orientation in an aluminum cup containing Tissue-Tek OCT freezing medium and snap frozen with liquid nitrogen. Longitudinal sections (6 μm) were cut at −19 °C, fixed in acetone, and subjected to indirect immunohistochemical staining.
[3]Vitiligo state, ''NV'', ''EV'', ''AV'', or ''CV'' means feather tips were from SL chickens that never developed SLV (n=3), from vitiliginous SL chickens (n=7) just (<2 weeks) before, during, or after SLV development.
[4]Data are shown as mean ± SE.
[a,b]Data in the same row without common letters are different at $P \leqslant 0.05$.
[5]T cells include both αβ and γδ TCR+ T cells.

lowest in NV samples, intermediate in EV and CV samples, and highest ($P \leqslant 0.05$) in AV samples (Table 1). On the basis of the proportions of IgM+ cells at the various states of SLV, the majority of infiltrating B cells (Bu-1+) appeared to be IgM+ cells (Table 1). Calculated T- to B-cell ratios (TCR+ cells vs. Bu-1+ cells) were lower ($P \leqslant 0.05$) in AV samples (1.52 ± 0.21) than in NV samples (5.16 ± 2.66) because of a relatively greater increase in B-cell than in T-cell infiltration in AV samples compared with other states of SLV (Table 1).

Examination of hematoxylin and eosin-stained formaldehyde-fixed feather sections confirmed the mononuclear nature of the leukocyte infiltrate and the notable absence of granulocytes, including heterophils (avian equivalent to neutrophils), at all vitiligo states (data not shown). In addition, natural killer cells do not appear to make up a significant portion of pulp-infiltrating lymphocytes in SLV based on flow cytometric analysis for CD8+ lymphocytes without surface CD3 (arbitrary definition of avian natural killer cells; (Gobel *et al.*, 1994)) in pulp cell suspensions from active autoimmune lesions (data not shown).

Profiles of cytokine expression throughout SLV development

Owing to limited availability of chicken cytokine-specific antibodies, cytokine expression was examined at the transcriptome level using quantitative reverse transcriptase PCR. Relative expression was calculated by the $\Delta\Delta C_t$ method (Wong and Medrano, 2005) and was expressed as fold change with respect to feather tips collected throughout the course of the study from NV SL chickens. As each vitiliginous chicken developed SLV at different weeks of age, to demonstrate the general trend of cytokine expression in SLV development and progression, data for each bird were aligned with respect to time of visible SLV onset (set as 0).

In feather tips collected from SLV chickens several weeks (>2) before SLV onset (BV), expression levels for all targets were similar to those of NV samples, with the exception of IL-8, IL-10, and IL-21, which exhibited ⩾3 fold higher levels than NV samples (Figure 3). In feather tips collected from SLV chickens closer to (⩽2 weeks before; EV) SLV onset, parallel increases in the relative expression of IL-8 and IL-10 could be observed, with IL-10 reaching higher than BV levels in samples collected 1 week (−1) before SLV onset (Table 2 and Figure 3). With SLV onset, expression of IL-8, IL-10, IL-21, and IFN-γ increased compared with BV samples, with all four cytokines simultaneously reaching peak levels and remaining elevated until 3 weeks post SLV onset (AV; $P<0.05$; Table 2 and Figure 3). This active phase of SLV was accompanied with significant, although much lower, increases in IL-1β, IL-6, IL-12β, IL-15, and iNOS (Table 2 and Figure 3). A notable exception to this expression pattern was IL-4, which did not increase above BV levels and exhibited the highest relative expression at much later time points than other targets (Table 2). With the progression of SLV, levels of most targets returned to BV levels, especially in white growing feathers collected from birds that exhibited complete depigmentation for at least 1 week (CV; Figure 3). Throughout the course of SLV development, from initiation, progression to complete pigmentation loss, the actual relative expression value of chemokine IL-8 and cytokines IL-10, IL-21, and IFN-γ, nevertheless, differed greatly between birds (Table 2). However, the combination of these cytokines, especially IL-10, IL-21, and IFN-γ, emerged as the signature cytokine profile during the early stage and active progression of SLV in all vitiliginous chickens. Expression of IFN-γ at the protein level was confirmed in a pilot study by indirect immunohistochemistry using mouse anti-chicken IFN-γ antibodies developed by Dr Lillehoj (Animal and Natural Resources Institute, USDA-ARS, Beltsville, MD; data not shown).

Relative expression levels for IL-12α and IL-17F could not be determined based on the formulae of the $\Delta\Delta C_t$ method, because no C_t value was detected in the calibrator sample. The relatively low expression of IL-12α and IL-17F in SLV can, however, be inferred based on calculation of the ΔC_t value (C_t of target—C_t of endogenous 28S control), which is a relative measure of target gene expression within a sample.

DISCUSSION

The SL chicken is an excellent animal model for human autoimmune vitiligo as suggested by the many phenotypic and etiological similarities between the two cases, which are summarized in Table 3. Although SLV is a multifactorial

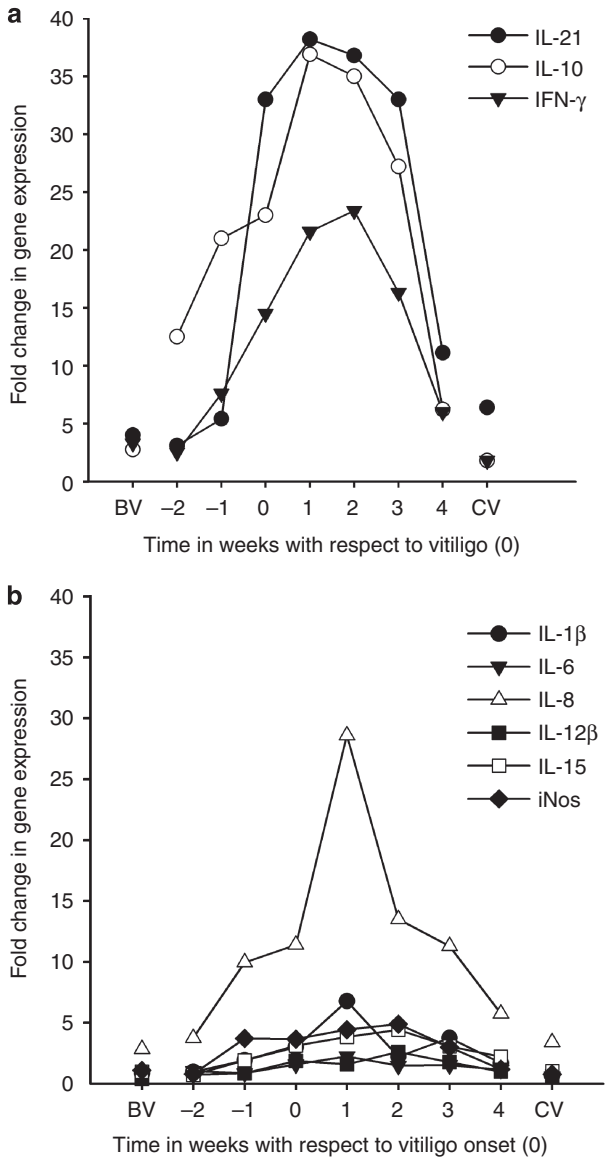

Figure 3. Time course of the relative expression of cytokines and inducible nitric oxide synthase (iNOS) in growing feathers collected from seven SLV chickens throughout vitiligo development. Relative expression was calculated by the $\Delta\Delta C_t$ method, using a cDNA pool made from growing feather of three SL chickens that never developed vitiligo as the calibrator and chicken 28S as the endogenous control gene. The *X*-axis represents the time (weeks) of feather tip collection with respect to vitiligo onset (0); BV and CV represent data from feathers collected from the seven SL chickens >2 weeks before vitiligo and >1 week after complete pigmentation loss, respectively; weeks −2 and −1 represent early vitiligo (EV) and weeks 0–4 correspond to active vitiligo (AV). (**a**) Expression pattern of IL-21, IL-10, and IFN-γ. (**b**) Expression pattern of IL-1β, IL-6, IL-8, IL-12β, IL-15 and iNOS. Also see Table 2.

Table 2. Mean of highest relative expression[1], mean time of highest relative expression, and times of significant elevation ($P \leqslant 0.05$) of cytokines and iNOS in growing feathers collected before and throughout the development of vitiligo in SL chickens

Target gene	Highest expression mean[2]	Time (weeks)[3]	Times elevated[4]
IFN-γ	31.82 ± 5.53	1.43 ± 0.43	0, 1, 2, 3
IL-10	56.26 ± 12.42	1.00 ± 0.53	−1, 0, 1, 2, 3
IL-21	56.49 ± 12.82	0.86 ± 0.46	0, 1, 2, 3
IL-1β	7.47 ± 1.91	1.57 ± 0.43	0, 1, 3
IL-4	2.60 ± 1.01	2.72 ± 0.78	None
IL-6	4.06 ± 1.59	0.43 ± 0.53	0, 1, 2, 3
IL-8	36.64 ± 9.48	1.14 ± 0.58	1
IL-12β	3.19 ± 0.82	1.14 ± 0.77	0, 2
IL-15	5.70 ± 2.15	2.00 ± 0.84	2
iNOS	7.61 ± 2.52	1.14 ± 0.55	1, 2

[1]Relative expression was expressed as fold changes with respect to expression levels in feather samples from three non-vitiliginous SL chickens (calibrator sample).
[2]Mean ± SEM of the seven SL chickens' highest expression level of each target gene during SLV development.
[3]Mean time ± SEM (n=7) in weeks (with respect to SLV onset; week 0) when highest expression occurred.
[4]Time in weeks with respect to SLV onset (week 0) when targets were significantly ($P \leqslant 0.05$) elevated compared with pigmented growing feathers collected from the seven SLV chickens more than 2 weeks before SLV onset (BV samples). Also see Figure 3.

disease in nature, the immune system was shown to have a critical role in SLV expression in susceptible chickens.

Humoral immunity was initially implied by immunosuppressive effects of bursectomy (excision of generative organ for B cells), which resulted in decreased incidence and severity of SLV in SL chickens (Lamont and Smyth, 1981; Boissy *et al.*, 1984). Findings from the current study also support a potential role of B cells, as their infiltration into the target tissue was associated with SLV development (Figure 2 and Table 1). The reason for B-cell infiltration and the role of B cells in the active SLV lesion is not clear from this study. B cells will follow similar infiltration signals as T cells and can participate in cellular immunity through antigen uptake and presentation, as well as antibody secretion. Considering the late and low expression of IL-4 (Table 2), a cytokine supportive of humoral immunity and isotype switching, together with the predominant IgM+ phenotype of infiltrating B cells (Table 1), it seems that IL-4 is not responsible for B-cell activities in this autoimmune response. Evidence for B-cell participation in vitiligo is also provided by the presence of melanocyte-specific autoantibodies in sera of vitiliginous SL chickens and vitiligo patients (Harning *et al.*, 1991; Austin *et al.*, 1992). Furthermore, a destructive effect of vitiligo antibodies on human melanocytes via complement-mediated and antibody-dependent cellular cytotoxicity was demonstrated *in vitro* and *in vivo* (Cui *et al.*, 1993; Gilhar *et al.*, 1995). From the current study, it is reasonable to speculate that autoantibodies and feather-infiltrating B cells are more important in amplifying than initiating melanocyte loss in SLV.

Phenotypic characteristics of infiltrating leukocytes and cytokine profiles in the current study confirm a more prominent involvement of cell-mediated, specifically Th1-mediated, than humoral immune activity in melanocyte loss

Table 3. Comparison between vitiligo in SL chickens (SLV) and in humans

		SLV	Human vitiligo	References
Main target tissue		Growing feathers	Skin	
Phenotype	Symptom	Depigmentation in growing feathers	Depigmentation in the skin	
	Associated autoimmune disorders	Autoimmune thyroiditis, alopecia-like feathering defect	Autoimmune thyroiditis and other disorders of immune origin	Erf, 2010; Spritz, 2010
	Onset	Early puberty to young adults (6–14 weeks of age)	Early puberty to young adults (10–30 years of age)	Smyth, 1989; Erf, 2010
	Severity	Erratic to complete	Erratic, occasionally complete	Smyth, 1989; Erf, 2010
Etiology		Multifactorial (genetic, immunological, metabolic, and environmental factors)	Multifactorial (genetic, immunological, metabolic, and environmental factors)	Erf, 2010; Boissy and Nordlund, 2011
Genetics	Vitiligo susceptibility genes	Multigenic, identification in progress	Multigenic, candidate genes identified	Wick *et al.*, 2006; Spritz, 2010
	Intrinsic melanocyte defects	Yes	Yes	Boissy *et al.*, 1986, 1991
Immunology	Target tissue infiltrating leukocytes	Macrophages, CD4, CD8, and B cells	Macrophages, CD4 and CD8 cells	Erf *et al.*, 1995; Le Poole *et al.*, 1996
	Melanocyte-specific autoantibodies	Yes	Yes	Harning *et al.*, 1991; Austin *et al.*, 1992
	Cytokines[1] and iNOS in the affected tissue	↑IFN-γ	↑IFN-γ	Wang, 2001; Grimes *et al.*, 2004
		↑IL-10	↑IL-10	Grimes *et al.*, 2004
		↑IL-6	↑IL-6	Moretti *et al.*, 2002, 2009
		IL-4 no change	IL-4 no change	Grimes *et al.*, 2004
		IL-17 low levels of expression	↑IL-17	Bassiouny and Shaker, 2011
		↑IL-21, ↑IL-1β, ↑IL-8, ↑IL-12β, ↑IL-15, and ↑iNOS	Not determined	
Metabolism	Oxidative stress	Yes	Yes	Schallreuter *et al.*, 1999; Erf *et al.*, 2005
Environmental factor	Viral association	Positive association with live HVT vaccination at hatch	Possible association with HCV and HIV	Erf *et al.*, 2001; Tsuboi *et al.*, 2006; Seyedalinaghi *et al.*, 2009

Abbreviations: HCV, hepatitis C virus; HVT, herpesvirus of turkey; iNOS, inducible nitric oxide synthase.
[1]The expression profile of cytokines and iNOS in the growing feather was determined in the current study.

as previously suggested (Wang, 2001; Wang and Erf, 2003; Shi *et al.*, 2009). Compared with the relative proportions of B (Bu-1 +) cells, those of T cells, including αβ and γδ TCR + cells, were numerically higher at all vitiligo states (Table 1 and Figure 2). Moreover, although both αβ and γδ T cells infiltrated, the infiltration of αβ T cells was proportionately greater during active SLV (Table 1), an observation consistent with previous reports (Erf *et al.*, 1995; Shresta *et al.*, 1997). Moreover, in line with previous reports on SLV (Erf *et al.*, 1995; Wang and Erf, 2004) and in vitiligo patients (Le Poole *et al.*, 1996; Le Gal *et al.*, 2001), the current study showed the predominance of CD8 + compared with CD4 + cells at all vitiligo states (Table 1). Predominance of cytotoxic lymphocytes in active SLV lesions, their close juxtaposition, and aggregation around apoptotic melanocytes (Wang and Erf, 2004), together with macrophage infiltration at early and active stages of SLV, further supports a Th1-mediated, melanocyte-specific cellular response in SLV. Macrophages, which are important immune system activators and effector cells of cell-mediated immunity, were also present in perilesional skin from vitiligo patients (Le Poole *et al.*, 1996).

IFN-γ, which is the signature cytokine of Th1-type cell-mediated immunity and was markedly increased in samples collected at and after SLV onset (Table 2 and Figure 3), may have a central role in the SLV pathomechanism. IFN-γ may function in several ways during SLV expression and progression. IFN-γ is important in macrophage activation, phagocytic activity, cytokine and chemokine production, MHC I and II expression, and NO production due to transactivation of iNOS (Abbas *et al.*, 2010). In the current study, increased expression of IL-1β, IL-6, IL-8, IL-12β, and iNOS indicates macrophage activation, which, because of the relatively low levels of macrophage infiltration, does not appear to be a major mechanism by which IFN-γ exerts its effects in SLV (Table 2, Figures 2 and 3). IFN-γ was shown to increase autophagosome formation in human CD4 + T cells (Son *et al.*, 2010) and macrophages (Shi and Kehrl, 2010). Autophagosomes were reported in melanocytes from SL

chickens that developed vitiligo (Boissy *et al.*, 1983, 1986), which may have been promoted by elevated levels of IFN-γ in the environment of the melanocyte. In addition, IFN-γ is able to stimulate the expression of MHC I and MHC II molecules on various types of cells. In the current study, the heightened and sustained proportions of MHC II-expressing cells in EV, AV, and CV samples compared with NV samples (Figure 2) are a strong indication of an immunologically active site, with IFN-γ supporting antigen presentation and macrophage, B-cell, and T-cell activation. Finally, IFN-γ has been shown to have inhibitory effects on Th17-cell development from CD4+ precursor cells (Kelchtermans *et al.*, 2008), which may explain the relatively low IL-17F expression and the absence of heterophils (avian equivalent to neutrophils) in feathers from vitiliginous SL chickens in the current study.

On the basis of the above discussion, IFN-γ appears to have an essential role in SLV pathogenesis. As IFN-γ expression was not preceded by IL-12 and IL-15 (two cytokines capable of IFN-γ induction) production, the question regarding the mechanisms driving IFN-γ expression needs to be addressed. Review of recent literature revealed important positive interrelationships between IFN-γ and IL-10 and IL-21, the other cytokines with marked expression in EV and AV samples (Table 2 and Figure 3). IL-10 is generally known as a cytokine with anti-inflammatory and immunosuppressive activities (Abbas *et al.*, 2010). IL-10 has, however, recently been shown in humans and mice to have immunostimulatory effects by increasing IFN-γ production and activation of natural killer cells and cytotoxic T lymphocytes (Shibata *et al.*, 1998; Lauw *et al.*, 2000; Tilg *et al.*, 2002). A pre-stimulated immune system and the presence of high levels of IL-10 are two important factors that favor immunostimulatory effects of IL-10. Both these preconditions are met in SLV. Marked elevation of IL-10 expression was observed in the current study, together with heightened inflammatory immune activities, such as mononuclear cell infiltration, expression of IL-8 chemokine, IFN-γ, and MHC II expression in EV and AV feather tips. The source of immune activation may be the routine vaccination at hatching with live herpesvirus of turkey against lymphoma-causing Marek's disease virus. Herpesvirus of turkey translocates to and infects the feather follicle epithelium (Cho, 1975), and has been identified as a reliable environmental trigger of SLV expression in susceptible SL chickens (Erf *et al.*, 2001). Recent cytokine gene expression analysis at the transcriptome level by Abdul-Careem *et al.* (2008) showed that herpesvirus of turkey administration in chickens resulted in a concomitant increase in IFN-γ and IL-10 expression in feathers reaching peak levels at 7 days and returning to levels of control by 10 days post vaccination.

IL-21, a cytokine with pleiotropic functions, is the target of intensive investigations partially because of its ability to induce differentiation and expansion of Th17 cells in chronic inflammatory diseases (Nurieva *et al.*, 2007). Low expression of IL-17F and the absence of heterophils in SLV lesions do not justify a pathological role of IL-21 through Th17 cells in SLV. IL-21 was also shown to be required for effector function and sustainability of CD8+ T cells, including cytotoxic activities and IFN-γ production, in chronic viral infection (Elsaesser *et al.*, 2009; Frohlich *et al.*, 2009; Yi *et al.*, 2009); inhibition of Foxp3+ Treg cell development, which indirectly enhanced activity of CD8+ T cells (Li and Yee, 2008); and induction of IL-10 expression in activated CD4 and CD8 T cells in a mouse model of systemic lupus erythematosus (Spolski *et al.*, 2009). Therefore, overexpression of IL-21 may have an underestimated role in the pathogenesis of SLV directly through its positive effects on cytotoxic T-cell function and IFN-γ expression, and indirectly through its support of immunostimulatory effects of IL-10.

Taken together, SLV is an autoimmune disease in which melanocyte loss is associated with mononuclear leukocyte infiltration consisting predominantly of T cells (with more CD8+ than CD4+ cells), followed by B cells and macrophages. Cytokine expression in the target tissue in the same individuals throughout SLV development revealed concomitant increases in the relative expression of IFN-γ, IL-10, and IL-21 before and during active melanocytes loss. The mechanisms underlying this cytokine signature and the interrelationship of immune cells and cytokines in melanocyte loss need to be further examined to gain insight into the etiology and pathology of the spontaneous expression of vitiligo in susceptible SL chickens.

MATERIALS AND METHODS

Experimental design

Animals. Twenty SL chicks, homozygous for the B101 MHC haplotype, were randomly selected from 20 families at the time of hatching, vaccinated with live herpesvirus of turkey (Fort Dodge Animal Health, Fort Dodge, IA), and assigned to one floor pen at the Arkansas Experiment Station Poultry Farm in Fayetteville, Arkansas. A standard rearing protocol was followed, and feed and water were provided *ad libitum.* Animal use was approved by the University of Arkansas Institutional Animal Care and Use Committee.

Feather collection

Samples (six feather tips per bird; Figure 1a) were collected weekly from chickens starting at 5 and ending at 18 weeks of age. Half of the feather tips were snap-frozen in Tissue-Tek OCT freezing medium (Sakura Finetek, Torrance, CA) and the other half were placed in RNA*later* (Qiagen Valencia, CA). All samples were stored at −80 °C until use. SLV was scored at each feather collection following previous criteria (Wang and Erf, 2003). Basically, SLV was given a score of 1 to 5 if the newest growth of 0%, ⩽20, >20 and ⩽60, >60 and ⩽99, and 100% of the growing feathers demonstrated visible depigmentation. Upon completion of sample collection, feather tips from seven SL chickens that developed SLV and reached a score of 5 during the 18-week period (SLV chickens) and three SL chickens that never developed SLV (NV chickens) were used in this study.

Indirect immunoperoxidase staining of growing feathers

Longitudinal sections (6 μm thick) were cut at −19 °C in a cryostat (CM3050-S, Leica Microsystems, Bannockburn, IL), processed and immunochemically stained as described in Erf *et al.* (1995). Primary antibodies were mouse mAbs specific for chicken TCR γδ (cTCR γδ), cTCRαβ1, cTCRαβ2, cCD4, cCD8, cBu-1 (B cells), cIgM, cKUL01

(macrophages), and cMHC II molecules (Southern Biotechnology Associates, Birmingham, AL).

Image analysis

Image analysis was carried out under a bright-field microscope (× 40 magnification), and ImagePro Plus software was used (Media Cybernetics, Silver Spring, MD). The amount of positively stained cells was expressed as the percentage of the whole tissue section (epidermis and pulp) analyzed (% area). All evaluations were made by the same person.

RNA isolation, quantification, and cDNA synthesis

For each chicken and time point, the three feather tips preserved in RNA*later* were homogenized by Tissue Tearor (BioSpec Products, Bartlesville, OK, Model: 985370-395) in lysis buffer provided in the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA was isolated from homogenates with on-column DNA digestion (Qiagen). RNA was eluted in 30 µl RNase-, DNase-free water and stored at −80 °C until use. RNA integrity and concentration were determined as previously described (Hamal *et al.*, 2010). RNA (175 ng per sample) was transcribed to cDNA in a 30 µl reaction volume using a High-Capacity cDNA reverse transcription kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The resulting cDNA was aliquotted and stored at −80 °C until analysis.

Relative expression of cytokines and iNOS

Primers and probes used in this study are available upon request. Real-time PCR was performed according to Hamal *et al.* (2010). The calibrator sample was a pool of cDNA prepared from feather tips of the three SL chickens without vitiligo (NV). The relative gene expression was determined by the $\delta\Delta C_t$ method (Wong and Medrano, 2005).

Statistical analysis

To determine the effect of SLV state, one-way analysis of variance (SYSTAT software, Chicago, IL) was conducted using the general linear model procedure, followed by Fisher's LSD multiple means comparison. For the time course of gene expression, EV, AV, and CV samples were compared with BV samples collected from the same SLV chickens. All data were reported as mean ± SE, and the differences were considered to be significant at $P \leqslant 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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